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(54) Partial Cationization of Protein-Containing Antigens and Method of Immunization and Desensitization

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(73) Same as inventor

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PARTIAL CATIONIZATION OF PROTEIN-CONTAINING ANTIGENS AND METHOD OF IMMUNIZATION AND DESENSITIZATION

This invention relates to a method of preparing a partially cationized protein-containing substance, such as an antigen, having increased antigenicity, to the product of this method, and to a method of enhancing immune response to a native antigen in a mammal.

The importance of the use of antigens in the prevention of infectious disease through immunization and in the treatment of allergies through desensitization is well known.

The basis of immunization is the exposure of the organism to be immunized to dead or weakened infectious agents (viruses, bacteria, toxins, etc.) or extracts thereof which contain a foreign, generally macromolecular substance which is capable of evoking an immune response. These substances are generally referred to as antigens. Likewise, allergic reactions can be lessened by desensitization, wherein such a substance is used to suppress the normal allergic response caused by foreign substances, referred to as allergens.

Most antigens and allergens are either wholly or partially composed of protein.

The action of antigens is thought to be dependent in part on the antigen's affinity for certain binding sites on cells of the living tissue affected by the antigen. Such tissue may be blood, internal organs, skin, eyes, etc. Interaction of the antigen to the binding site stimulates the production of antibodies which in turn defend the organism against infectious agents containing the antigen.

Because antigens are foreign substances, they can have adverse side affects on the organism sought to be immunized. This is most important in the treatment of humans and livestock. It is therefore desirable to be able to achieve an effective immunogenic response by utilizing lower levels of the antigen. If antigenicity can be increased, it necessarily follows that a smaller dosage can be administered to achieve a given level of immunization.

One way to increase the antigenicity of a substance is by using an adjuvant in conjunction with the antigen. An adjuvant is a substance which augments the immunogenic response by aiding the antigen's interaction with the living tissue. Examples of adjuvants include alum salts for human use. A problem which militates against the use of adjuvants in <u>in vivo</u> immunization is the toxicity and the side effects these compounds induce. Because of this, adjuvants are generally disfavored for use in humans or in any other organism where toxicity and/or side effects are a concern. The elimination of the need for adjuvant cooperation is therefore desirable.

Some foreign substances do not evoke an antigenic response at all, or do so very poorly, when brought into contact with living tissue. One theory is that such substances lack the threshold amount of binding capability or strength to cause such a response. It would be advantageous to be able to convert such substances from a non-antigenic form to an antigenic form. This would allow vaccines to be produced which could elicit an immunogenic response to a substance where this was not previously possible.

Immunization methods are normally carried out by subcutaneous or intramuscular injection of the vaccine. Oral intake of an antigen is not feasible in most instances because it often causes suppression of rather than increase in the immune response. Exceptions to this general rule include live attenuated bacterial and viral vaccines. Oral vaccines offer advantages such as lower expense and ease in administration and packaging. Therefore, it would be better to be able to administer vaccines in oral form without loss in immunogenic effect.

The chemical modification of antigens, particularly protein-containing antigens, has been known for some time in fields of research, such as cell surface labelling, see D. Danon, Use of Cationized Ferritin as a Label Of Negative Charges On Cell Surfaces, J. Ultrastructure Research, Vol. 38, pp. 500-510 (1972).

Prior methods of antigen cationization have taught the complete cationization of the antigen molecule. Here "complete cationization" is intended to mean that a given substance has had all its groups amenable to cationization so altered. Completely cationized antigens are generally unsuitable for in vivo use due to their

excessive reactivity.

Nakamura et al. Jpn. J. Nephrol, 28(1), 37-44 (1986) disclose chemical cationization of bovine serum albumin.

Muckerheide et al., Fed. Proc., 45(3), abstract No. 55 (1986) disclose that cationized bovine serum albumin induces an antibody response.

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There has heretofore been no recognition in the prior art that cationized antigens can be used in in vivo treatment and prevention of disease by use as vaccines or desensitization agents. Furthermore, the prior art has not recognized that partially cationized antigens can be used for this purpose. Indeed, it has been reported that completely cationized antigenoproteins do not exhibit altered immunological properties (9).

It is an object of the invention to provide a partially cationized antigenic protein-containing substance, and a method for the preparation thereof, which can be administered orally, and which does not require an adjuvant.

SUMMARY OF THE INVENTION

As used herein, "protein-containing substance" includes all proteins, as well as substances whose molecular makeup is in some part proteinacious, such as lipoproteins and proteosaccharides. These may be substances which do or do not have antigenic properties in their native forms. More specific examples of said substances include, without limitation, bovine serum albumin (BSA), hen egg albumin (OVA), bovine gammaglobulin (BGG), ferritin, bacterial endotoxin, viral proteins, diptheria toxin and tetanus toxoid, and killed microorganisms, including bacteria and viruses and protein-containing products thereof.

As used herein, cationization means the conversion or substitution of functional groups on the protein portion of the protein-containing substance whereby the substance is rendered relatively more cationic. Such functional groups are normally anionic within a physiologic pH range and are converted to or substituted for a cationic or nonionic moiety. An example of such a cationization is the reaction whereby anionic side chain carboxyl groups are substituted with polycationic aminoethylamide groups.

As used herein, the unreacted or "native" form of the protein-containing substance is indicated by a prefix "n" and the cationized form is indicated by a prefix "c". For example, bovine serum albumin (BSA) may be expressed as either "nBSA" as the native form, or "cBSA" as the cationized form.

According to one aspect of the present invention there is provided a protein-containing substance, for use as a vaccine or a densensitization agent in the treatment or prevention of

disease, said substance being partially cationized through the attachment of at least one agent selected from primary amines, secondary amines and tertiary amines, wherein

a) said partially cationized protein-containing substance has an isoelectric point, as measured by isoelectric focusing in a polyacrylamide gel, less than 9.5; and

b) the immunological response of an animal to the partially cationized protein-containing substance is greater than to the unmodified, native protein-containing substance.

According to a further aspect of the present invention there is provided the use of a partially cationized antigen in the manufacture of a preparation for enhancing the immune response of an animal to an unmodified, protein-containing antigen, said antigen being partially cationized through use of an agent selected from the primary amines, secondary amines and tertiary amines so that

a) the isoelectric point of the modified antigen is less than 9.5; and

b) the partially cationized antigen induces in said animal a stronger immunological response than the unmodified antigen.

According to the invention there is provided a partially cationized antigenic protein-containing substance having increased antigenicity as compared to the same native protein-containing substance, the partially cationized substance having an isoelectric point less than 9.5 and preferably from 6.5 to 9.5, determined by isoelectric focusing as hereinafter described.

The invention also provides such a partially cationized protein-containing substance for immunizing a mammal. Administration can be effected orally or parenterally, with or without an adjuvant.

A preferred method of partial cationization involves use of a reagent comprising at least one carbodiimide and at least one amine. Control of the degree of cationization is effected by varying the pH, time parameters and concentration of the reactants. The reaction may be halted or quenched readily at a desired degree of partial cationization, in a manner known in the art. By way of non-limiting example, BSA reaction with a carbodiimide and amine mixture may be quenched after substitution of about 20 new amino groups in place of anionic carboxyl groups. A fully cationized BSA would contain about 80 new amino groups.

In general, an addition or substitution of from about 20% to about 60% of the theoretical maximum possible number of amino groups will result in an isoelectric point ranging between about 6.5 and 9.5 as determined by isoelectric focusing.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The protein-containing substance (PCS) can be cationized by several methods known in the art (15, 9). The preferred method is the reaction of ethylenediamine/ 1-ethyl-3-(3-dimethyl amino propyl)-carbodiimide (EDC) with the PCS. This particular reaction involves the activation of the carboxyl groups of a protein with carbodiimide and the subsequent reaction of the activated carboxyl with a nucleophile of the general type $+R-NH_2$ to obtain the primary amine type derivatives. Considerable versatility can be achieved since both the chemical nature of the modification, i.e. introduction of primary, secondary or tertiary amine groups and the degree of modification of the protein carboxyls can be varied by proper choice of reagents, reaction time and pH of the coupling reaction.

The cationization methods suitable for use in the present invention are those which effect cationization under mild reaction conditions. As used herein mild reaction conditions are those under which the molecular character (e.g. 1°, 2° and 3° structure) of the subject PCS is not substantially altered so as to adversely affect its antigenic pH immunogenic character. These conditions can be most generally described as under relatively low temperature and low ionic strength as well as neither very acidic (e.g. below pH 4) nor very basic

(e.g. above pH 8) nor presenting a very oxidative or reductive environment.

The pH of the EDC reaction is generally maintained in the range of from about 4.5 to about 6.8. More rapid substitution of carboxyl groups in the EDC reaction occurs at the lower pH levels within this general range. For example, BSA cationized at pH 6.0 for 30 minutes migrated during electrophoresis the same distance as BSA cationized at pH 4.75 for 15 minutes (i.e., 0.7cm).

Reaction time is determined by the concentration of the reactants and by the degree of cationization desired. The preferred reaction time is in the range of from about 5 minutes to no more than two hours.

The reaction is maintained within the general range of from about 4° C to about 37° C and is generally maintained at about 25° C.

Each known method of cationization may be halted or quenched according to several methods known in the art. The EDC reaction is quenched with a buffer, preferably an acetate buffer, which halts the reaction. Concentration of the acetate buffer is about 4M.

Cationization can be verified and quantified using known gel electrophoresis as hereinafter described, and isoelectric focusing techniques known in the art. (14) Ninhydrin reagent can also be used to determine the number of amino groups substituted in a protein molecule as shown hereinafter.

It is preferred that the protein-containing substance be cationized to an extent whereby it exhibits

increased antigenic character such as an increased immunogenic character or an increased allergic response suppressing character. Such increases may be determined by methods known in the art such as those described below. Although not a limitation to the scope of the applicability of the invention, for most types of PCS, this will be at a point where the isoelectric point falls within a range of from about 6.5 to about 9.5.

Also as a nonlimiting guide, most PCS types which are modified to such an extent so as to increase their antigenic character will generally have from about 20% to about 60% of the maximum possible number of those anionic groups amenable to modification under "mild" conditions while that PCS is in its native state, so modified. As used herein "modified" is intended to mean any type of chemical modification which causes cationization of the PCS.

Although most of the PCS types modified according to the inventive method fall into the above-described isoelectric point range, it is within the skill in the art to determine and adjust the degree of partial cationization which increases the antigenic character for all PCS types including those PCS types, in native or cationized form, whose isoelectric points may fall outside this range.

The following Examples exhibit the inventive method as practiced on several antigens. Variations of the parameters and methodology for optimization of the method for any specific PCS is within the known art.

Example 1

Bovine Serum albumin (nBSA), five times crystallized, was cationized according to the general procedure described by Border (9).

Five grams of nBSA was dissolved in distilled water to a volume of 25 ml and admixed with a solution of 67 ml ethylene diamine in 500 ml distilled water. The pH of this solution was adjusted to about 4.75 with 6N HCl. To this was added 1.8 grams of 1-ethyl -3-(3 dimethyl amino propyl)-carbodiimide.

The reaction was permitted to react for varying periods of time with constant stirring, while the temperature was maintained at about 25° C and the pH was held constant. After quenching with 4M acetate buffer mixture, the reaction was subjected to multiple dialysis treatments against distilled water and lyophilized. It was then passed through a column of Sephadex G-25 and lyophilized again before use.

EXAMPLE 2

The method of Example 1 with the exception that the pH of the reaction was adjusted to about 6.0 with 6N HCl and the reaction was permitted to react for about 1 hour.

EXAMPLE 3

The method of Example 1 with the exception that the PCS may be native bacterial endotoxin instead of nBSA.

Example 4

The method of Example 1 with the exception that the PCS used was native tetanus toxoid instead of nBSA (purchased from Lederle Laboratories, Pearl River, NY).

Example 5

The method of Example 2 with the exception that the PCS used was native hen egg albumin (OVA) instead of nBSA.

Example 6

The method of Example 1 with the exception that the PCS used was ferritin (purchased from Sigma Chemical Co., St. Louis, MO), samples of which were cationized for 5 minutes, 15 minutes and 30 minutes.

Example 7

The method of Example 1 with the exception that the PCS used was heat killed E.coli bacteria.

Example 8

The method of Example 1 with the exception that the PCS used was bovine gamma globulin (BGG), purchased from Sigma Chemical Co., St. Louis, MO.

Example 9

The method of Example 1 with the exception that fluorescein isothiocyanate (FITC), a small

non-immunogenic molecule, was conjugated either to an nBSA carrier or to a cBSA carrier (as prepared in Example 1). These conjugates were used as the PCS. The fluorescein isothiocyanate-BSA conjugation method is as follows:

FITC is used in 0.5 mg concentration per mg of protein. FITC is added to BSA solution and the pH is adjusted to about 8.4 with borate buffer. The conjugation is allowed to proceed for about 1 hour with continuous mechanical stirring at slow speed.

The suspension is dialyzed in a cold room with frequent changes of saline adjusted to a pH of about 7.8 with borate buffer. The dialysis requires several days and is complete when the dialyzate is virtually free of yellow-green color under ultraviolet light. The conjugate is clarified by centrifugation.

Other hapten-carrier conjugations may require more complex chemical reactions to achieve covalent bonding between the reacting molecules.

Cationization Assay: Agrose Gel Electrophoresis

Materials:

1) Corning® Electrophoresis Agarose Universal Gel Film (1% Agarose, 5% sucrose, 0.035% EDTA, in 0.065M barbital buffer, pH 8.6);

2) Corning® Universal PHAB Buffer (Sodium barbital 17.7g, barbital 2.6g, sodium chloride 1.0g, disodium EDTA 0.7g, and sucrose octaacetate) Reconstituted with distilled water to 0.05M buffer with 0.035% EDTA, pH 8.6.

3) Corning® Amido Black 10B Stain

NOTE: Above purchased from Fisher Scientific.

4) Corning® Cassette Electrophoresis Cell with Corning Power Supply.

METHODS:

1) 1.0 µl of sample at a concentration of 30 mg/ml is loaded onto gel into each well. (For sample of lesser concentration, 1 µl of sample is loaded and allowed to dry between applications until appropriate concentration is reached).

2) 95 µl buffer is added to each side of cell.

3) Gel is placed on electrophoresis unit and run for appropriate time period. (For cBSA - time period is 40 minutes; nBSA is run as a control).

4) Gel is removed from electrophoresic unit and placed in amido black stain for 15 minutes. Gel is destained in 5% acetic acid for 20 seconds.

5) Gel is allowed to dry.

6) Gel is destained again in 5% acetic acid until good contrast is seen between back and background.

7) Gel is rinsed in 2 separate distilled H_2O baths.

8) Gel is again allowed to dry.

Samples may be subjected to isoelectric focusing to determine the isoelectric point (pI) value of the particular cationized product, by a conventional procedure described in the prior art.

Results:

Chemical Properties and Electrophoresis

The cationization procedure results in the substitution of anionic side chain carboxyl groups by polycationic aminoethylamide groups (14, 15). The products are readily soluble in water and show a single band on gel electrophoresis in the presence of sodium dodecyl sulfate, with or without a reducing agent such as mercaptoethanol. The band migrates more rapidly toward the cathode than the native BSA monomer. Passage through Sephadex G 200 shows a single peak which appears slightly before the nBSA peak.

Electrophoresis on agarose gel (Table 1) shows that the two hour reaction time produces a molecule which appears to have attained a maximum positive charge in the case of cBSA since preparations which are permitted to react for a longer period of time do not exhibit increased migration toward the cathode.

The following results were obtained using the above outlined agarose gel technique:

Electrophonesis Results

Reaction Time	Distance Migrated Toward Cathode (cm)		
	cBSA	cFerritin	cOVA
15 min.	0.7	1.0	3.0
30 min.	1.0	1.3	4.0
60 min.	1.6	1.7	5.0
90 min.	1.9	N/T	5.3
120 min..	2.1	N/T	5.5
150 min.	2.1	N/T	N/T
180 min.	2.1	N/T	N/T

N/T = Not Tested

The products were determined to have an isoelectric point greater than 6.5 and up to about 9.5. The isoelectric point is used as one of the measures to determine the degree to which a given PCS has been cationized.

Amino Group Determination Using Ninhydrin Reagent

The number of amino groups substituted in a protein molecule by cationization can be determined by means of ninhydrin reagent. Ninhydrin reagent, purchased from Sigma Chemical Co., was used to prepare a curve with increasing concentrations of glycine, which served as a standard. An experimental procedure with BSA was as follows:

Ninhydrin reagent was added to a cBSA dilution and to nBSA (control), and the mixtures were heated at 80°C for 20 minutes, at which point the color of each mixture

was read on a spectrophotometer at 550. A BSA sample, cationized for 15 minutes with ethylene diamine/EDC and quenched with an acetate buffer, showed a gain of 20 new amino groups over the native BSA control. Fully cationized BSA should contain about 80 new amino groups. The isoelectric point of the BSA sample cationized for 15 minutes was 8.0. It is thus possible to determine quantitatively the number of amino group substitutions on a molecule of a protein-containing substance.

Such a determination is useful in expressing the degree to which a protein-containing substance has been cationized. The degree of cationization may thus be expressed as a percentage of those anionic groups amenable to substitution under relatively mild conditions (i.e. the reaction conditions of the inventive cationization method disclosed herein) while the protein-containing substance is in its native state, which are substituted to effect cationization. This percentage, though not all inclusive, is usually in the range of from about 20% to about 60%.

Immunogenicity and Desensitization Studies.

Mice: BDF_1 and BALB/c mice. 6-8 weeks of age, were purchased from the Jackson Laboratory, Bar Harbor, ME.

Adjuvants: Complete and incomplete Freund's Adjuvant (IFA) and bacterial lipopolysaccharide were purchased from Difco Laboratories, Detroit MI. Aluminum hydroxide gel was prepared in our laboratory according to the method of Levine and Vaz (11) or was in the form of commercial Maalox* (Rorer Inc., Fort Washington, PA).

* Trade-mark

Antibody Assays: A quantitative ELISA technique was used as previously described (12). Standard curves were run each time an assay was performed, using known amounts of antibody raised against either native or cationized BSA. The sera were then assayed on antigen-coated ELISA plates. The coatings used on plates varied in some tests and these variations are described in the Tables below, where applicable.

T cell proliferation assays: BDF_1 mice were injected in the hind footpads and tail base with 100 µg native or cationized antigen emulsified in Incomplete Freud's Adjuvant. The inguinal and popliteal lymph nodes were removed 10 days later and the subsequent cell suspension was passed over a nylon wool column as has been described (13). The nylon wool non-adherent cells were then resuspended in complete RPMI 1640 medium containing 10% horse serum, 1mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM glutamine, 5×10^{-5} M 2-mercaptoethanol, 25 mM HEPES and 5 µg/ml gentamycin, and plated in 96 well flat bottom plates (Costar, Cambridge, MA) at 5×10^{-5} cells/well. Native or cationized antigen were added at various concentrations in serum-free complete RPMI 1640 to triplicate wells. Serum-free medium served as a control. Cells were incubated at a final volume of 200 µl at 37°C with 5% CO_2 for 72 hours at which time 1 µCi ^{3}H-thymidine was added to each well. Cells were harvested 20 hours later using a Skatron harvester and radioactivity was determined by liquid scintillation spectrophotometry.

The following results were obtained with cBSA cOVA cFerritin, cE.Coli bacteria, cTetanus Toxoid, cBGG and FITC-

cBSA conjugate, in immunogenicity and desensitization studies comparing their antigenicity to that of their native forms.

Table A

Immunization	Anti BSA Antibodies (µg/ml) 9d	14d	21d
50 µg n BSA, i.v. injection 5 times	58	28	0
50 µg cBSA, i.v. injection 5 times	393	450	425

cBSA prepared as in Example 1.

Tested on BSA plate.

Table A shows that cBSA is immunogenic when injected into mice.

Table B

EFFECT OF CATIONIZATION ON IMMUNOGENICITY

OF BOVINE SERUM ALBUMIN

Time of Cationization	Immunogenicity-Antigen Specie	Relative µg/ml Antibody
None	nBSA	350
15 min.	cBSA	1220
30 min.	cBSA	1450
60 min.	cBSA	1135
120 min.	cBSA	110

Anti BSA response µg antbs/ml at 14d).

Cationization was conducted at a pH of 4.75 and at a temperature of 25°C.

Table B shows that the length of time of protein cationization controls its immunogenicity. Cationization

optimizes immune responsiveness. However, if cationization is excessive, immunogenicity is sharply reduced, if not totally eliminated. Administration was intraperitoneal (i.p.) with alum adjuvant (1 mg/dose).

Table C

EFFECT OF CATIONIZATION ON ANTIGEN REACTIVITY (BOVINE SERUM ALBUMIN)

Time of Cationization	Antigen Specie	Relative Antigen Activity
None	nBSA	++
15 min.	cBSA	+++
30 min.	cBSA	++++
60 min.	cBSA	+++
120 min.	cBSA	-

BSA primed T lymphocytes responsiveness to antigen

Table C shows that too extensive cationization of BSA creates a molecule incapable of stimulating BSA primed T lymphocytes.

Cationization for a time up to 60 minutes greatly enhances the ability of BSA to stimulate lymphocyte proliferation, but excessive cationization creates a non-immunogenic or poorly immunogenic molecule.

Table D

Effect of in vivo pretreatment with soluble nBSA or cBSA on the IgG antibody response to either antigen

Group	PRETREATMENT d -9,8,7 (intravenous)	IMMUNIZATION (intraperitoneal)	% Enhancement OR SUPPRESSION[1] 10d	14d	21d
A	100 µg nBSA	100 µg nBSA in alum	-18	-26	-51
B	100 µg nBSA	100 µg nBSA in IFA[2]	-95	-94	-92
C	100 µg nBSA	100 µg cBSA in IFA	-84	-87	-91
D	100 µg cBSA	100 µg cBSA in alum	2200	6055	400
E	100 µg cBSA	100 µg cBSA in IFA	98	113	28
F	50 µg cBSA	100 µg cBSA in IFA	184	241	42

[1] Compared to control groups immunized with nBSA or cBSA and pretreated with physiological saline. Suppression indicated by negative value.

[2] Incomplete Freund's Adjuvant

d = day

cBSA was prepared as in Example 1.

Table D shows that cBSA evoked greater immune response following initial pretreatment with cBSA whereas nBSA actually suppressed the production of anti BSA antibodies.

Table E

Effect of in vivo pretreatment with doses of soluble cBSA on the immune response (IgG) to either antigen

Group	PRETREATMENT d -9,8,7 (intravenous)	IMMUNIZATION	% ENHANCEMENT OR SUPPRESSION[1] 10d	21d
A	25 µg cBSA	100 µg nBSA	259[2]	413
B	25 µg cBSA	100 µg cBSA	497	274
C	10 µg cBSA	100 µg nBSA	867	2818
D	10 µg cBSA	100 µg cBSA	566	683
E	1 µg cBSA	100 µg nBSA	230	866
F	1 µg cBSA	100 µg cBSA	1321	1018
G	25 µg nBSA	100 µg nBSA	-85	-27
H	25 µg nBSA	100 µg cBSA	-22	NS[3]
I	10 µg nBSA	100 µg nBSA	-87	NS[3]
J	10 µg nBSA	100 µg cBSA	NS	NS
K	1 µg nBSA	100 µg nBSA	NS	NS
L	1 µg nBSA	100 µg cBSA	NS	NS

[1] Immunizations were given intraperitoneally using 1 mg alum as adjuvant.

[2] Compared to control groups immunized with nBSA or cBSA and pretreated with physiological saline. Suppression indicted by negative value.

[3] NS = not significant (less than 5% change).

Table E shows results of a more detailed study of the immune response to BSA obtained with small quantities (1-25 mg) of the cationized BSA and the resulting immune response, both in terms of increase in enhancement and the longevity of the increase.

Table F

EFFECT OF ORAL ADMINISTRATION OF ANTIGEN ON IgG RESPONSE

Feeding (3 x 20 mg)	Immunization (100 µg) in alum	Anti BSA Response - IgG (µg/ml) 10d	14d	24d
-	BSA	900	812	240
-	cBSA	1024	2076	3050
BSA	BSA	87	58	57
cBSA	BSA	2700	1074	875
BSA	cBSA	250	287	675
cBSA	cBSA	3700	6300	6300

Tested on BSA plate

Table G

EFFECT OF ORAL ADMINISTRATION OF ANTIGEN ON IgE RESPONSE

Feeding (3 x 20 mg)	Immunization (100 µg) in alum	Anti BSA Response - IgE PCA titer 10d	14d	24d
-	BSA	40	82	80
-	cBSA	80	160	160
BSA	BSA	20	20	40
BSA	BSA	20	20	20
BSA	cBSA	10	10	10
cBSA	cBSA	20	20	20

Tables F and G show the effect of oral administration of the cationized and native form of the antigen. Table F records the increase in anti-BSA IgG levels resulting from cBSA immunization, while Table G shows that cBSA suppresses anti-BSA IgE levels.

Table H

EFFECT OF ORALLY ADMINISTERED cOVA
ON IMMUNE RESPONSE TO nOVA AND cOVA

Feeding with 20 mg Antigen	Intraperitoneal Immunization with 0.1 µg Antigen in 1 µg Alum	Immune Response Anti OVA at 14d (µg/ml)
None	nOVA	140
None	cOVA	765
nOVA	nOVA	35
nOVA	cOVA	180
cOVA	nOVA	1320
cOVA	cOVA	2250

OVA cationized for 1 hour as described in Example 5.

Table H shows that feeding with nOVA suppresses immune response to OVA. In contrast, feeding (oral administration) with cOVA enhances greatly the immune response to both nOVA and cOVA.

Table I

IMMUNE RESPONSIVENESS TO cOVA AND nOVA in BDF_1 MICE

Antigen Administered i.p. with 1 mg Alum	Response Days After Immunization (µg/ml Anti-OVA Antibodies) 9d	14d	21d
0.1 µg nOVA	80	120	120
1.0 µg nOVA	450	840	660
10 µg nOVA	1250	1420	1300
0.1 µg cOVA	425	976	770
1.0 µg cOVA	1300	2010	1660
10 µg cOVA	1500	2350	3200

OVA cationized as described in Example 5.

Table I shows cOVA is far more immunogenic than nOVA most notably at lower doses of antigenic challenge.

Table J

EFFECT OF INTRAPERITONEALLY ADMINISTERED CATIONIZED FERRITIN IN BDF_1 MICE

Bled at days	Percent enhancement c Ferritin over n Ferritin (control) 5 min. cF	15 min. cF	30 min. cF
9	169	97	217
15	134	101	490
21	122	123	241
35	195	206	457

Cationized Ferritin, cF, was produced as described in Example 6, and administered i.p. to BDF_1 mice. Antibody levels were determined from time-interval bleeds measured by ELISA on plates coated with native ferritin.

Table J shows a substantial increase in immunogenicity of partially cationized ferritin.

Table K

EFFECT OF I.p. ADMINISTERED CATIONIZED E coli BACTERIA IN BDF_1 MICE

No. of bacteria injected	Percent enhancement over controls		
	10 days	20 days	30 days
1×10^6	160	210	180
5×10^6	220	450	650
1×10^7	260	500	460
5×10^7	320	800	650

Heat killed E. coli bacteria were cationized as in Example 7 and washed 3 times with saline and injected i.p. into mice, and the mice were later bled. Antibody concentration was determined by ELISA in which plastic plates were coated with untreated bacteria. Antibacterial antibody titers in mice immunized with untreated bacteria served as controls and were compared with antibody titers from mice immunized with partially cationized bacteria.

Table K shows a substantial increase in immunogenicity of partially cationized bacteria.

Table L

EFFECT OF I.p. ADMINISTERED CATIONIZED TETANUS TOXOID IN BDF_1 MICE

Immunization i.p. with 1 mg alum	Anti-tetanus antibody Percent enhancement cTT over nTT (control)		
	14 days	28 days	35 days
1 ug cTT	220	300	320
10 ug cTT	325	560	720
100 ug cTT	280	450	660

Tetanus Toxoid was cationized as described in Example 4.

Animals were bled at various time intervals and antibody levels were determined by ELISA on native TT.

Table L shows that partial cationization increases the immunogenicity of tetanus toxoid.

Table M

EFFECT OF I.p. ADMINISTERED CATIONIZED BGG IN BDF_1 MICE

Immunization i.p. with 1 mg alum	Anti-BGG antibodies µg/ml 9 days	14 days	21 days
nBGG			
1 µg	55	80	60
10 µg	110	400	800
50 µg	350	850	1000
100 µg	320	1200	1200
cBGG			
1 µg	110	180	180
10 µg	450	1200	1600
50 µg	1200	2500	2500
100 µg	850	2100	2600

Bovine Gamma Globulin (BGG) was cationized as described in Example 8.

Animals were bled at various time intervals and antibody levels were measured by ELISA. Column purified anti-BGG antibodies were used as standards. Each group comprised 5 mice, and the entire experiment was repeated twice.

Table M demonstrates that partial cationization greatly increases the immunogenicity of Bovine Gamma Globulin.

Table N

EFFECT OF FITC CONJUGATED TO cBSA IN BDF_1 MICE

Bled at days	percent enhancement FITC-cBSA over FITC-nBSA (control)
10	240
15	280
22	330
30	180
37	160

FITC-BSA conjugates were prepared as described in Example 9.

Immune response was determined by measuring antibodies produced against FITC-nBSA or FITC-cBSA in mice on ELISA plates coated with FITC-KLH conjugate.

Table N shows that a small non-immunogenic molecule, a hapten such as FITC, becomes highly immunogenic when conjugated to a partially cationized protein (cBSA).

The above data establish that the method of immunizing a mammal or suppressing allergic response in a mammal in accordance with the invention includes administering an effective amount of a partially cationized antigenic protein having an isoelectric point ranging from about 6.5 to about 9.5, the administration being either oral or parenteral, and with or without an adjuvant. A method of enhancing immune response to a native antigen in a mammal comprises administering parenterally to a mammal an effective amount of that antigen which has been partially cationized such that its isoelectric point ranges from about 6.5 to about 9.5, and thereafter administering parenterally to the mammal an amount of that native antigen sufficient to evoke an immune response.

Although the invention is in no way limited by any theory as to why beneficial results are achieved, it is postulated that cationized proteins may be more immunogenic for the following reasons:

1. Increased affinity for antigen presenting cells (APC).

2. Alterations in the antigen processing by the APC.

3. More efficient recognition by T helper cells.

4. Greater affinity for self recognition antigens (Ia).

5. Greater affinity for T cell receptors.

6. Activation of a new type of T helper cells with more efficient recognition of the antigen.

The above examples are for illustrative purposes only.

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A protein-containing substance, for use as a vaccine or a densensitization agent in the treatment or prevention of disease, said substance being partially cationized through the attachment of at least one agent selected from primary amines, secondary amines and tertiary amines, wherein

a) said partially cationized protein-containing substance has an isoelectric point, as measured by isoelectric focusing in a polyacrylamide gel, less than 9.5; and

b) the immunological response of an animal to the partially cationized protein-containing substance is greater than to the unmodified, native protein-containing substance.

2. The partially cationized protein-containing substance according to claim 1, having an isoelectric point in the range from 6.5 to 9.5.

3. The partially cationized protein-containing substance according to claim 1 or claim 2 wherein the unmodified protein-containing substance is selected from bovine serum albumin, hen egg albumin, bovine gamma globulin, ferritin, bacterial endotoxin, viral proteins, tetanus toxoid, diphtheria toxin, and killed microorganisms and protein-containing products thereof.

4. The partially cationized protein-containing substance according to claim 1 or 2, being conjugated with a hapten, and wherein the immunological response of an animal to the hapten,

conjugated to the partially cationized protein-containing substance, is greater than to the hapten, conjugated to the unmodified native protein-containing substance.

5. The use of a partially cationized antigen in the manufacture of a preparation for enhancing the immune response of an animal to an unmodified, protein-containing antigen, said antigen being partially cationized through use of an agent selected from the primary amines, secondary amines and tertiary amines so that

a) the isoelectric point of the modified antigen is less than 9.5; and

b) the partially cationized antigen induces in said animal a stronger immunological response than the unmodified antigen.

6. The use of a partially cationized antigen according to claim 5, wherein the partially cationized antigen has an isoelectric point in the range 6.5 to 9.5.

7. The use of a partially cationized antigen according to claim 5 or claim 6, wherein the unmodified antigen is selected from bovine serum albumin, hen egg albumin, bovine gamma globulin, ferritin, bacterial endotoxin, viral proteins, tetanus toxoid, diphtheria toxin, and killed microorganisms and protein-containing products thereof.

8. The use of a partially cationized antigen according to

claim 5 or 6, wherein the partially cationized antigen is conjugated with a hapten, and wherein the level of antibodies produced by the animal to the conjugated hapten/partially cationized antigen is greater than to the hapten conjugated with the unmodified antigen.

9. The use of a partially cationized antigen according to claim 5 or 6 wherein the cationization is effected by coupling the agent to the antigen through an amide bond.

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ABSTRACT OF THE DISCLOSURE

A method for preparing an antigenic protein-containing substance by partially cationizing a native protein-containing substance to an isoelectric point ranging from about 6.5 to about 9.5. The partially cationized substance exhibits increased antigenicity as compared to the native protein-containing substance and is useful in mammalian immunization and desensitization treatments by oral or parenteral administration.